A STUDY OF THE PHYSIOLOGICAL RELATIONS OF SCLEROTINIA CINEREA (BON.) SCHRÖTER

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Introduction

This paper reports the results of an experimental study regarding certain physiological activities of the brown-rot fungus of stone fruits. The investigation concerns itself primarily with the conditions influencing the penetration and infection of green and ripe fruits by the fungus in question, the action of the parasite on the host cell, and the secretion of the enzymes which act upon the cellulose and pectic substances of the host. The work was undertaken with the hope of throwing some further light upon the factors concerned in fungous parasitism. Our present conception of this subject is based upon fragmentary and, in some respects, contradictory evidence. However, each year there are acquired new facts, or new applications of known facts, bearing upon this exceedingly involved and complex question. An examination into the history of investigations concerning the interaction of host and parasite shows that the study of this subject dates back to the work of the pioneers in plant pathology; modern methods and recent discoveries have, however, given an added impetus to research along this line.

Progress in combating fungous diseases depends not only upon a familiarity with the life history of the parasite, but more especially upon an intimate knowledge of the metabolism of the parasite and the nature of the changes which it induces in the host. Indeed, many of our recommendations for controlling parasitic diseases of plants will perhaps be modified when a more exact knowledge of the interrelations of host and parasite is gained. Furthermore, a more intimate knowledge of the physiological aspects of plant pathology will undoubtedly throw much light on the question of immunity and susceptibility.

We should, of course, like to know more about the factors favoring or inhibiting parasitic action, as well as the conditions

which influence the infection and the penetration of parasitic fungi. It would also be interesting to know why some fungi are so virulent and rapid in their destructive action on the host; for instance, it would be instructive to know whether it is due to the secretion of an enzyme, or a toxic substance (e. g., some acid), or to the disturbance of the osmotic relations of the host cells, or to some other perhaps unknown factor. For a study of some of these problems the writer has chosen as the organism *Sclerotinia cinerea* (Bon.) Schröter, the fungus causing the brown rot of stone fruits. This form is particularly suitable for the purpose since it is a virulent parasite, yet grows well as a saprophyte —readily lending itself to cultivation in the laboratory.

Historical Review

Space will permit only a brief review of some of the more important papers dealing with certain aspects of this subject. Much of the literature that is indirectly concerned with the problem, or that is fully reviewed or superseded by subsequent publications, will not be discussed here.

In the period from 1858 to 1878 little experimental evidence appeared concerning the nature of the action of fungous parasites, although several writers make mention of the penetration of host cells by fungous hyphae. Penetration was then frequently spoken of as merely a process of boring through ("durchbohrung") the host tissue, Kühn (34), as early as 1858, mentioning this fact in a discussion of the potato-blight fungus. A few years later, in 1863, de Bary (1) speaks of the penetration of the host by *Peronospora*, and further makes mention of this fact in connection with his work on the rusts (2); again in his work 'Morphologie und Physiologie der Pilze, Flechten, und Myxomyceten' (3) he discusses the penetration of the host, but says he has no knowledge of the force that causes this boring into the host tissue.

Hartig (26), in his early work on wood-destroying fungi, as well as in his later investigations, emphasizes the fact that fungi are able to destroy cellulose. By a microscopical study of diseased wood he found that the properties of the latter are very materially changed by the fungus; he did not, however, attempt to isolate an enzyme.

De Bary (4), in 1886, gives us the first important contribution to our knowledge concerning the action of parasites on host cells. This author, in his epoch-making research on the fungus now known as *Sclerotinia libertiana*, reports that the organism secretes a substance that discolors, plasmolyzes, and finally kills the host cells. This toxic secretion penetrates the host cells in advance of the fungus, killing them before they are actually pierced by the fungous filaments. De Bary was able to isolate this toxic substance, which he considered as probably an enzyme, and found that it would cause an injury to the host tissue similar to that produced by an attack of the fungus itself. He holds that the fungus will not grow on living tissues, for it attacks only through a wound and kills the cells in advance of itself, thus not actually growing upon the living tissue. The product resulting from the disintegration of the cell wall of the host was thought to be a sugar that served as food for the fungus. In this connection de Bary also mentions finding oxalic acid encrusting the older fungous filaments.

The next important paper on the interaction of host and parasite was that of Marshall Ward (51) published just two years after de Bary's work and concerning itself with a species of *Botrytis* causing a lily disease. In this excellent piece of work the author showed that the fungous hyphae on coming in contact with such solid substances as sections of a lily bulb, or even a cover glass, secrete from the tips drops of a substance that has a very peculiar effect on the host cell. He found that a water extract of this secretion when applied to sections of a lily bulb will cause the cell walls to swell and to assume an abnormal appearance; the middle lamella is first dissolved and finally the entire cell wall is disorganized. Ward does not consider that this toxic secretion is stimulated by starvation.

Several investigators have held that the penetration of many fungi is due to chemotropism, i. e., that penetration of the fungous hyphae is due to some stimulus which the constituents diffusing slowly from within the host cells exert. Büsgen (16), Miyoshi (39), Behrens (6), Schmidt (44), and others have adhered to the view that chemotropism is important, but more recent work, such as that of Fulton (25), does not uphold the theory.

Behrens (6) investigated some of the physiological relations of saprophytes in comparison with parasites, using *Mucor stolonifer*, *Penicillium sp.*, *Botrytis cinerea*, and *Oidium* (=*Sclerotinia*[1]) *fructigenum*. This author holds that *Sclerotinia* does not produce a cellulose-dissolving enzyme, and that the fungus merely forces its way through the host tissue by a purely mechanical force, or that, in some cases, it splits the middle lamella but does not dissolve it. In the case of the other fungi mentioned above he believes that an enzyme is secreted which dissolves the middle lamella. The cause of the injury due to *Sclerotinia*, he holds, is not that the cellulose walls or the pectin of the middle lamella is dissolved, but that the turgor and the osmotic relations of the penetrated cells are materially modified. According to this author some substance diffuses through the walls and stimulates the fungus to bore through or between the cell walls. He demonstrated in *Botrytis* and *Penicillium*, moreover, a thermo-stable toxic body which disintegrated the host cells, and believes that these fungi secrete a pectin-dissolving enzyme which is different from that which acts upon cellulose.

Nordhausen (40), at about the same time, made similar studies on *Botrytis cinerea* and comes to similar conclusions. He finds that the enzyme does not cause a strong swelling of either the middle lamella or the cellulose cell walls, the action in this respect being more like that of de Bary's *Sclerotinia*. Smith (46) studied the parasitism of *Botrytis cinerea*, but in certain particulars did not get the same results as de Bary and Ward. Like them he finds that the parasite secretes some soluble substance that penetrates and kills the living cells in advance of the fungous filaments, but unlike Ward he could detect no swelling of the cell wall. Smith believes that this toxic substance is not an enzyme, for boiling does not inactivate it, but thinks that it is perhaps oxalic acid, since this substance is always present in the cultures and amounts in some cases to as much as two per cent. The analytical methods whereby the oxalic acid was determined, unfortunately, are not given.

Schellenberg (43) investigated the action of several saprophytic and parasitic fungi on hemicelluloses from a number of

[1] Wehmer, C. Ber. d. deut. bot. Ges. **16**: 298–307. 1898; Saccardo, Syll. Fung. **4**: 34. 1886.

different sources. He claims that these fungi act differently toward different celluloses, dissolving some and having no effect on others. The nature of the penetration and the action of certain parasites on the host tissue were also studied. There was no case in which *Botrytis* dissolved true cellulose, but it readily dissolved the hemicellulose part of the cell, leaving the cellulose intact. According to this author, therefore, the penetration and dissolving action of such parasites as *Botrytis vulgaris* is due to their ability to dissolve hemicelluloses. He considers that the middle lamella is largely composed of hemicelluloses or closely allied substances. According to this view, therefore, organisms that dissolve the middle lamella are essentially hemicellulose-dissolving forms. As a result of his studies on *Sclerotinia fructigena* and *S. cinerea*, Schellenberg finds a different action on different fruits, but in no case does he report a splitting of the cells along the line of the middle lamella, as some previous investigators have reported. He believes that there is a slight dissolving action on that part of the cell wall which is in immediate contact with the fungous filament, but that the rest of the cell wall remains intact. In the twigs also he finds that the fungus dissolves the hemicellulose and leaves the true cellulose unacted upon.

An extensive literature has developed concerning the enzymes of importance in the nutrition of fungi, but since these investigations either deal with saprophytes, or are only indirectly concerned with the work to be reported in this paper, it will be unnecessary to do more than mention some of the papers here. Among the more important contributors may be mentioned Ward (50, 52), who was the first to use pure cultures of a wood-destroying fungus (*Stereum*), Biffen (9), who studied the biology of *Bulgaria polymorpha*, Bourquelot and Hérissey (13), who investigated the enzymes in sporophores of *Polyporus sulphureus*, Czapek (18), who made his investigations with natural infections of *Merulius lacrymans* and with other fungi, Kohnstamm (33), who worked on some species of *Merulius*, Buller (14, 15), who investigated sporophores of *Polyporus squamosus*, Van Iterson (28), who developed methods for isolating cellulose-dissolving bacteria and fungi, and Dox (19), who investigated the enzyme action of species of *Penicillium* and *Aspergillus*. It is interest-

ing to note that although we have every reason to believe that cytase is present in timber-decay organisms yet its presence has been demonstrated only indirectly by cytological methods. It is true, however, that many of the investigators mentioned above who found no cytase used the sporophores in their experiments and not the mycelium.

The status of the subject of the enzymes concerned in the metabolism of parasitic fungi is given in Reed's recent publication (42), which concerns itself with the enzymes produced by the parasitic fungus *Glomerella rufomaculans*. This author has proved that the parasite produces many of the enzymes that had previously been reported for saprophytes, and by quantitative methods has demonstrated different enzymes acting on the several classes of nutritive substances, such as carbohydrates, glucosides, fats, and proteins. He did not, however, investigate the cytolytic activity of the fungus but states that the nature of the diseased host would indicate that cytase very probably is not produced by this fungus. Peltier (41), as a result of his investigations with *Botrytis Fuckeliana*, finds that the host cells are killed in advance of the fungous penetration, and that the parasite secretes a thermo-stable toxic substance, but, unlike Smith, finds no oxalic acid. The method of testing for oxalic acid unfortunately is not given.

The action of bacteria on cellulose and other plant products has been extensively studied by a number of investigators, but for the purpose at hand it will suffice to cite some of the more recent publications in which the earlier literature is reviewed. The work of Jones (29, 30), which gives a good resumé of the early work on this subject, is reviewed below under the discussion of pectin.

McBeth and Scales (38) report that a number of bacteria and fungi hydrolyze cellulose and claim that filamentous fungi play a very important rôle in the destruction of cellulose in soils. The cellulose-destroying fungi, according to these authors, act differently toward different kinds of cellulose, but their experiments do not seem to support this conclusion. Kellerman and McBeth (32) have also contributed to our knowledge of the cytolytic activity of fungi. Kellerman (31) has employed a method

by which it is demonstrated that cytase diffuses in agar considerably beyond the region of hyphal penetration, and that a portion of the agar containing the enzyme dissolves cellulose in a manner similar to that of the fungus itself.

The organism employed in my work was isolated from an infected plum twig, at Madison, Wisconsin. The original cultures were taken from a single colony in a Petri dish, this procedure giving reasonable assurance that I was working with a single strain of the organism. Regarding the systematic relations of this organism a word may not be out of place here, since considerable confusion has arisen in the literature regarding the specific name of the organism causing the brown rot of stone fruits (27, 53, 37). Woronin (56) has made an important contribution designed to establish the systematic position of the two species *Sclerotinia cinerea* and *S. fructigena*. It has generally been held that *S. fructigena* causes the brown rot of stone fruits in this country, while in Europe this fungus is found only on pome fruits; but Matheny (37) has recently given good evidence tending to show that it is *S. cinerea* which causes the brown rot of stone fruits both in this country and in Europe.

Experimental Studies

INFECTION

Some investigators, as, for instance, Zschokke (57), have held that *Sclerotinia cinerea* is unable to penetrate sound fruit, while Smith (45), among others, has held that the fungus rapidly penetrates and infects sound and unwounded fruit (peaches). Casual observation in the field would seem to justify the former view, for those fruits in contact with other fruits or twigs, and therefore liable to puncture or abrasion, are the ones that are usually found infected; indeed, field observations and laboratory experiments point to the conclusion that infection takes place much more readily, especially with immature fruits, when the cuticle is broken. One would, therefore, naturally raise the question as to whether or not infection can take place when the cuticle is unbroken, and if so under what conditions and in what stages of the development of the fruit. During the summer of 1913

the writer performed a number of experiments which throw more light on the question of the infection of the host.

Methods and Results.—The methods employed were as follows: Plum twigs bearing leaves and fruit were broken off and brought into the laboratory, washed with a mercuric chloride solution (1–1000) and in sterile water. They were then suspended in sterile moist chambers prepared by placing moistened absorbent cotton in the bottom of wide-mouthed one-liter Erlenmeyer flasks that had previously been plugged and sterilized. Twigs having one or more green leaves were used in every case, for in this way green plums hang on the twigs and remain alive for some time. This method was especially applicable here, for it enabled one to maintain absolutely sterile conditions in a moist atmosphere and at the same time keep the host living and in a normal condition. The results of these infection experiments are given in table 1.

Discussion of Results.—From these results it is evident that plums were infected as early as June 27, at which time they were immature, in fact not more than half-grown. Infection did not take place when a spore suspension was placed on very green and immature plums unless the epidermis was broken or punctured. There were, however, some instances where plums remained healthy in the flask for two or three weeks and became infected only after the lapse of time had brought about an artificial maturity. On the other hand, plums that were approaching maturity, though not mature, as well as mature fruits, may be infected by applying a spore suspension to the natural surface, i. e., a surface which has not been punctured or injured in any way. In this connection it should be mentioned that infection was much more readily accomplished when two plums were hanging so as to be in contact with each other than when they were not touching. This, no doubt, was due to the fact that a drop of water containing spores may be held between the plums long enough for spore germination and infection to take place. These results also indicate that infection takes place readily without puncturing when a portion of the mycelial felt is laid on the surface of either green or ripe fruit.

It should be noted here that one can sometimes find plums in the field only half-grown which are affected with the brown-rot

TABLE I

RESULTS OF INFECTION EXPERIMENTS WITH SCLEROTINIA CINEREA

Date	Fruit	Inoculating material	Treatment of surface	Method of inoculation	Results
June 27	Green plums	Spore suspension	Cuticle killed by steam	Surface application	++*
June 27	Green plums	Spores	Skin punctured with needle	Needle puncture	+
July 2	Green plums	Spores	Skin punctured with needle	Needle puncture	++
July 8	Green plums	Spores	Skin punctured with needle	Needle puncture	++
July 8	Green plums	Spore suspension	Untreated	Surface application	−
July 8	Sour cherries	Spores	Skin punctured with needle	Needle puncture	+
July 23	Green plums	Spores	Skin punctured with needle	Needle puncture	++
July 23	Green plums	Spore suspension	Untreated	Surface application	−
July 23	Green plums	Spore suspension	Skin cut	Surface application	++
July 23	Ripe plums	Mycelium	Untreated	Surface application	++
July 23	Green plums	Mycelium	Untreated	Surface application	++
July 30	Green plums	Mycelium	Untreated	Surface application	++
Aug. 5	Green plums	Spore suspension	Untreated	Surface application	+
Aug. 13	Nearly ripe plums	Spore suspension	Untreated	Surface application	++
Aug. 13	Ripe plums	Spore suspension	Untreated	Surface application	++

*++ indicates that practically every inoculated fruit became infected.
+indicates that only a portion of the inoculated fruits became infected.
− indicates that none of the inoculated fruits became infected.

fungus, but so far as the writer's observation indicates, infection in these cases takes place through the twig, or, in some cases, through another plum with which it is in contact and which in turn is infected through the twig. Nevertheless, field observations also verify the laboratory work in that plums (especially certain varieties, such as Wood) when approaching maturity may be infected in the field without being in contact with other fruits and without having any visible punctures or wounds in the skin. All these experiments and observations point to the conclusion that penetration of the cuticle is a very important factor in the infection of fruits, especially immature fruits; that infection of very green fruits without punctures is rare; and, on the other hand, that maturing fruits without punctures may be readily infected both by spores and by a mycelial felt in the field and in the laboratory.

PENETRATION

The nature of penetration and the course of the hyphæ of parasitic fungi in piercing host tissue is an interesting and important question in connection with a study of the nature of parasitic action. In the case of the brown-rot fungus growing on the plum it is of importance to know whether or not the hyphæ merely follow the middle lamellæ or whether they enter the cells wherever they come in contact with them. Previous investigators differ very widely in their opinions as to the nature and course of the penetration of the fungus in question, a condition which is perhaps partly explained by the fact that different hosts were employed in the various investigations. Furthermore, it appears that the methods employed in some of the researches were not of such a character as to readily yield complete information concerning all the facts in the case.

In my own work a study of the penetration of the host tissue by the fungus was made by examining a number of sections of infected tissue in which the disease had reached various stages of development, and comparing them with sections of healthy tissue from the same fruit. For this purpose a special method was employed.

Methods and Results.—Small pieces of fruit composed of diseased and sound tissue were cut from plums inoculated with

a pure culture of the fungus. These segments were immersed in 70 per cent alcohol just long enough to partially kill the fungous filaments and the host cells, yet not long enough to discolor the sound tissue or to modify or change the color of the diseased tissue in any way. From this material razor sections, containing both diseased and healthy tissue, were made, stained for a short time in eosin, and then partially destained with alcohol. If the pieces of plum had not remained in the alcohol for a sufficient length of time, the razor sections were immersed in 70 or 95 per cent alcohol before staining. By employing this method it is possible to stain the fungous filaments deeply, while the host tissue remains unaffected. Indeed, this method permits of a rather sharp color differentiation between the healthy and the diseased tissue, the latter being blackened by the disease. This method, though quite applicable for the purpose at hand, was primarily developed for another purpose, which will be discussed below.

Since every fungous filament is very sharply differentiated, one may readily study the course of the hyphæ with reference to the host cells. By staining, sectioning, and examining diseased material taken from the margin of the infected area, one finds the fungous hyphæ penetrating the cells at any point of contact; indeed, after examining a number of specimens by the method reported above, the writer finds no indications that the fungous hyphæ follow the middle lamellæ, as has been reported by other investigators (57, 6) for pears and other fruits. The above method also enables one to contrast the cell walls of infected and penetrated cells with those of normal tissue. It is entirely possible that the fungous filaments, on coming in contact with a cell wall, secrete just enough enzyme to dissolve their way through the cell walls, leaving the walls of the host cells surrounding the hyphæ entirely normal, i. e., without swelling or disorganization.

Another and somewhat different experiment was performed to get additional evidence on this point. From sound plums which had previously been rendered sterile by washing in bichloride of mercury solution (1–1000) and sterile distilled water, free-hand sections were cut with a razor sterilized in 50 per cent alcohol. The sections were arranged in hanging drop cultures

and each inoculated with a drop of a very dilute spore suspension containing two or three spores per drop. The progress of the fungus and the condition of the host cells were noted from day to day but no visible disintegration of the cell walls could be observed, nor did the fungus show any particular affinity for the middle lamellæ.

Conclusions.—We would conclude, therefore, as a result of direct observation on the host tissue, that the fungus penetrates the host very readily and rapidly, that it does not necessarily follow the middle lamellæ in the plum and the peach, and that there is no visible general disintegrating action on the middle lamellæ or on the cell walls of the living host.

ACTION OF THE FUNGUS ON THE LIVING HOST CELLS

A significant fact in the metabolism of the brown-rot fungus is that it induces such an exceedingly rapid decay in the infected fruits. This rapid decay might be connected both with a rapid growth of the fungus and with a pronounced power which the organism possesses of breaking down and changing the constituents of the host. Moreover, several representatives of the genus *Sclerotinia* have been reported to have the power of secreting an enzyme or some other substance which kills the host cells in advance of penetration. Were this the case, it would be expected that rapid decay would accompany the action of the parasite. Is this view applicable to the action of *Sclerotinia cinerea?* The investigators who have made a study of this organism differ very widely in their views regarding the effect which it has on the host tissues, and it seemed desirable, therefore, to determine the relation of hyphal penetration to the death of the cells.

Methods and Results.—In order to fix the material for this study, it was found satisfactory to proceed as follows: Small pieces of the host tissue were taken from the margin of the diseased area and placed in 95 per cent alcohol for a short time. Free-hand sections were made of this material so as to include both diseased and healthy cells, and the sections stained for a short time in eosin and subsequently decolorized in part with alcohol, if necessary to give the desired contrast. By this

method the fungus may be distinctly differentiated from the host tissue, the killing and staining agents having little or no effect on the host cells. There is a more or less sharply differentiated line of demarcation between the injured and the sound cells, as indicated by the darker color of the former. The effect of the fungus is readily discerned by the blackening of the host tissue, this being especially noticeable in green plums. The discolored and poisoned cells are not at first plasmolyzed, and it is to be noted here that discoloration rather than plasmolysis should be taken as the index of the toxic action of this fungus on its host. It should perhaps be mentioned here, too, that the blackened cells shade off somewhat gradually into the hyaline healthy ones, and that, therefore, there is not always a sharp line of demarcation between the diseased and the healthy cells. However, in spite of these difficulties, I was convinced, after having examined a large number of sections of diseased and healthy tissue, that there is no positive evidence that the host cells are discolored, and therefore injured and poisoned, in advance of actual penetration by the fungus.

The indirect method employed to determine the same point consisted in applying to sound fruits an extract from decayed plums. Fruits were disinfected with mercuric chloride solution, washed in sterile distilled water, and inoculated with *Sclerotinia cinerea*. When the plums had become thoroughly decayed the juice was extracted and filtered under sterile conditions through a Chamberlain filter. The juice thus obtained was incubated for one week at a temperature of 22–25° C., and also tested on nutrient agar plates, and found to be sterile by both methods. From sound plums, which had been disinfected in the usual manner, a cone-shaped plug was cut out and the resulting cavity filled with this sterile extract,—the controls being prepared in a similar manner, using sterile water instead of the plum extract. The results were negative, that is, the controls were not unlike those treated with the extract from decayed plums.

The same experiment was repeated in a modified form by using thin razor sections of both green and ripe plums, the sections being made under sterile conditions as before, and observed in a hanging drop of sterile juice from decayed plums.

By means of this method one could readily observe any changes that might take place in the cells and make accurate comparisons with controls. Frequent observations were made, and throughout this experiment, which continued for several days, one could not distinguish between the appearance of those sections in a drop of sterile water and those in the sterile extract from decayed plums. It is possible and perhaps probable that this fluid, being merely the juice of the fruit, was too dilute to be effective, but the experiment was made because of the possibility of positive evidence.

Discussion of Results.—The initial stage in the injury caused by this fungus is shown by discoloration only and not by plasmolysis, and therefore one cannot draw conclusions with absolute certainty as to the poisoning effect of the extract on the cells of a cut surface, for the latter turn brown as soon as exposed to the air, just as when infected with the organism. It was comparatively easy, however, to observe that the extract had no effect on the cell walls, for no difference could be observed between the cell walls of the tissue thus treated and those of the control specimens. Even where the sections were left in the extract for several days neither swelling nor disorganization of the cell walls or middle lamellæ was noted. When sections of plum tissue were inoculated with one or more spores of the brown-rot fungus no cell-wall disintegration resulting from the growth of the fungus could be observed. A comparative study of sections of tissue, respectively exposed and not exposed to the action of the extract from decayed fruit, showed that no difference could be detected between the two, and that, therefore, no enzyme with a perceptible cytolytic action exists under these conditions. It has been held by some, notably by Behrens (6), that the injury to the host cell is largely physical in that the fungus penetrates at such a prodigious rate that the fluids of the host cell are allowed to escape with loss of turgor to the latter; furthermore, that the osmotic equilibrium is soon destroyed, with plasmolysis and death ensuing. It is very probable that part of the rapid injury to the host can be explained on purely physical grounds, but this may not be the only factor involved, although we do not now know what chemical activity of the fungous cells may be concerned in the rapid killing of the host tissue.

ACTION OF THE FUNGUS ON CELLULOSE

A number of investigators have regarded cellulose dissolution as a very important factor in the parasitism of many fungi; indeed, some of the earlier workers seemed to consider this the prime factor involved. While it is a well known fact that there are many fungi, especially saprophytes, which hydrolyze, or dissolve, certain celluloses, research extending over a wide field has revealed the nature of parasitism to be a very complex one in which other factors are as important as the dissolution of cellulose and the cell wall.

It has been the writer's purpose to study from two different points of view the action of the brown-rot organism on celluloses, (1) by observing the action of the fungus on pure cellulose isolated from the host tissue, and (2) by studying microscopically its action on the host cell walls themselves. In the former study cellulose agar was used, the cellulose being isolated from plums by the methods discussed below.

Methods and Results.—In the above mentioned study of the action of the fungus on pure cellulose, a variety of reagents, media, and methods for the preparation of cellulose were employed, a brief account of which follows. Schweizer's reagent was prepared by adding a slight excess (40 grams to the liter) of copper carbonate to dilute ammonium hydroxide solution composed of three parts of water to ten parts of ammonium hydroxide (sp. gr. 0.90). The copper solution was then shaken vigorously, allowed to stand over night, and the supernatant solution siphoned off. This is the procedure employed by McBeth and Scales (38).

Paper cellulose from filter paper was prepared according to the method given by McBeth and Scales (38) by dissolving 15 grams of sheet filter paper in Schweizer's reagent, diluting about ten times with water, and precipitating the cellulose with a solution of one part of hydrochloric acid to five parts of water. This mixture was then further diluted to 15 or 20 liters, the supernatant liquid siphoned off, and the residue washed repeatedly with water until the precipitated cellulose was free from both copper and chlorine. After standing quietly for several days the clear liquid was siphoned off and the precipitate used for the preparation of cellulose agar.

Cellulose agar was made by adding about one per cent (estimated by the weight of the paper before treating with Schweizer's reagent) of precipitated paper cellulose, prepared as stated above, to a mineral nutrient solution, the complete medium having the following composition:

Cellulose suspension	500 cc.
Agar	10 grams.
Monopotassium phosphate, 1 gram Magnesium sulphate, 1 gram Sodium chloride, 1 gram Ammonium sulphate, 1 gram Calcium carbonate, 2 grams Tap water, 1000 cc.	500 cc.

The insoluble precipitate appearing in the mineral nutrient solution was filtered off before the cellulose suspension and agar were added. Good results were also obtained by using 0.5 gram of calcium nitrate instead of 2 grams of calcium carbonate, in which case filtering is unnecessary. The mineral nutrient solution having the composition tabulated above will be referred to as nutrient "A."

Another nutrient solution very low in organic matter was also employed in the cellulose agar, but with rather unsatisfactory results. This solution, which will be referred to as nutrient "B," is that employed by Reed (42), and is made up as follows, the only organic material present being the small amount of sodium citrate:

Ammonium nitrate	10 grams
Dipotassium phosphate	5 grams
Magnesium sulphate	1 gram
Sodium citrate	1 gram
Tap water	1000 cc.

In making the cellulose agar this nutrient solution was used in exactly the same way as nutrient "A."

Since previous investigators have held that the celluloses from various sources differ in their resistance to hydrolyzing enzymes, an attempt was made in this investigation to prepare a cellulose from a natural host—plums—of the parasite. In order to secure a cellulose that is modified as little as possible in the process

of isolation three different methods were employed in preparing cellulose from plums, the resulting products being designated, for convenience in reference, respectively as soda cellulose, washed cellulose, and potassium chlorate cellulose.

In the preparation of soda cellulose ripe plums were squeezed through cheese cloth and the pulp was washed thoroughly with water. The pulp was then treated with an 8 per cent solution of sodium hydroxide and heated in the autoclave at ten pounds pressure. After thoroughly washing the pulp with water the heating with alkali was repeated and the product given final washings until free from alkali.

The second method of isolating cellulose—washed cellulose—consisted in washing the fruit pulp with water until free from substances soluble in cold water. Water was then added and the mixture heated in the autoclave at 15 pounds pressure, and washed. The operation was repeated as long as any water-soluble substances could be detected. This method, of course, gives an impure cellulose, yet the product is one that is free from water-soluble substances.

The third method consisted in oxidizing, dissolving, and washing out the plum pulp until a pure cellulose—potassium chlorate cellulose—was obtained. Pulp, secured from ripe plums in the manner stated above, was washed with cold water until the wash water was free from solutes, and then treated with a cold solution composed of 30 grams of potassium chlorate dissolved in 520 cc. of cold nitric acid (sp. gr. 1.1). This mixture was kept in the ice box for about three weeks, at the end of which time the pulp was entirely white. This method[1] is said to yield a product that differs only very slightly from the original cellulose.

The product obtained by these various methods was not allowed to dry, for it is possible that drying changes the nature of cellulose so that it is more resistant to the action of cytolytic enzymes. A part of the cellulose obtained by each of the preceding methods was treated with Schweizer's reagent and precipitated with hydrochloric acid and washed as stated above under the preparation of filter-paper cellulose. These three cellulose preparations thus treated with Schweizer's reagent, as

[1] Fowler, G. J. Bacterial and enzymatic chemistry. 159. 1911.

well as the three corresponding untreated portions, were used in the preparation of cellulose agars, according to the method given above. The media were placed in test tubes of very small (8 mm.) diameter, and sterilized. The tubes of melted agar were then cooled rapidly in cold water in order to bring about the hardening of the agar before the cellulose had had time to settle to the bottom of the tubes.

Tubes of the various cellulose agars were inoculated with *Sclerotinia cinerea* and others with a species of *Penicillium*, which will be designated as *P. expansum*[1], isolated from decaying peaches and apples. Since these two fungi, viz., *Sclerotinia cinerea* and *Penicillium expansum*, act very differently toward the host, a word contrasting their action may not be out of place here. As a result of inoculating apples, peaches, or pears with a pure culture of *Sclerotinia* the host tissues are promptly killed, while the fruits remain practically as firm after complete decay as before inoculation. On the other hand, the fruits inoculated with the *Penicillium* become very soft and watery, developing a pustule or sunken area where the infection took place. One may assume, therefore, that the *Sclerotinia* does not materially affect the celluloses and pectic substances that make for the firmness of the fruit, while, on the other hand, *Penicillium* does affect these substances, causing the fruit to lose its firm consistency. Since these two fungi show such entirely different and opposing characteristics as regards their effect on the same host, it is interesting to compare their action in pure cultures on cellulose and pectin-like substances. Such a comparative study was made, the results of which are given in table II.

Discussion of Results.—The results given in table II indicate that both *Sclerotinia cinerea* and *Penicillium expansum* exhibited in general a very slight hydrolytic action when grown on cellulose isolated from the plum, there being very slight action with both fungi on the soda cellulose and also on the potassium chlorate cellulose and no action on the washed plum cellulose. On the other hand, both fungi very readily dissolve filter-paper

[1] A culture of this organism was sent to Dr. Chas. Thom, who very kindly examined it and gave as his opinion that it was *P. expansum*, or perhaps a strain of that species. The organism in question, when grown on the media employed by Thom, showed characters very similar to those of *P. expansum*, as given by Thom (48).

TABLE II

ACTION OF SCLEROTINIA CINEREA AND PENICILLIUM EXPANSUM ON CELLULOSE

Type of cellulose used	Nutrient solution added	Sclerotinia cinerea		Penicillium expansum	
		Growth	Cellulose hydrolysis	Growth	Cellulose hydrolysis
Soda cellulose	A	++†	−†	++	+
Soda cellulose	B	++	−		
Potassium chlorate cellulose	A	++	+	++	+
Potassium chlorate cellulose	B	++	+		
Washed ligno-cellulose*	A	+	−		
Washed ligno-cellulose*	B	—	−		
Washed cellulose	A	+	−	+	−
Soda cellulose (Schweizer's)	B	+	−	+	−
Soda cellulose (Schweizer's)	A	++	+		
Washed cellulose (Schweizer's)	A	+	−		
Soda cellulose	Peach juice	+++	−	+++	−
Filter paper strips	Peach juice	+++	−	+++	−
Filter paper strips	A	++	−	++	−
Filter paper strips	B	+	−	++	−
Filter paper strips	0.5% glucose solution	+++	−	+++	−
Filter-paper cellulose	A	++	+++	++	+++

*Ligno-cellulose is the name here given to cellulose from the vascular tissues of the plum, i. e., that part of the pulp which did not go through the cheese cloth.

†Growth and cellulose hydrolysis are indicated by +, the relative intensities of growth and degrees of hydrolysis being indicated by one or more + marks. Absence of growth and absence of hydrolysis are indicated by —.

cellulose, and, strange to say, *Sclerotinia* is just as active in this respect as *Penicillium*. In many cases the growth was as good on the plum cellulose as on the filter-paper cellulose, yet the hydrolytic action of the fungi was very much weaker on the former medium. No cellulose hydrolysis occurred where peach juice or some soluble carbohydrate, such as glucose, was added. It seemed probable at first that a very small amount of glucose, or peach juice, or sodium citrate would give the fungus a vigorous start and thus accelerate its cyto-hydrolytic activity, but the quantities of these substances employed was sufficient to exert a protective influence, there being a vigorous growth but no apparent cellulose hydrolysis.

The fact that these fungi do not dissolve cellulose, derived either from the host or from paper, when other organic nutrients are supplied, verifies the writer's observation that *Sclerotinia cinerea* does not disintegrate the cell walls of the host tissues. Furthermore, the fact that the fungus dissolves paper cellulose very readily when it is the only carbohydrate supplied, leads one to conclude that the action of the fungus on paper cellulose in a nutrient solution low in carbohydrates is not necessarily a good criterion for judging the behavior of the fungus in the host tissue. In the host tissue there may be a form of cellulose different from that of paper, and it is furthermore very evident that there is present in the fruit an abundance of organic material evidently operating in a protective manner. The fungus fails to produce cytolytic enzymes when grown on plum or paper cellulose to which peach juice or even a very little sugar has been added, but acts vigorously on paper cellulose to which no organic nutrient has been added. It is rather peculiar that both fungi act much more readily on paper cellulose than on cellulose isolated from the fruits which are natural hosts for these organisms.

Sclerotinia cinerea grows very slowly when first transferred to a nutrient medium poor in soluble carbohydrates, very few spores and no aërial mycelium being produced. At the expiration of a week or more one may observe that the fungous mycelium has penetrated the surface layer of the agar, and at the expiration of two to three weeks, in case the fungus is growing on paper-cellulose agar, a clear translucent ring may be observed

in the agar just below the fungous filaments, thus indicating that the cellulose is being hydrolyzed. With increasing age of the fungus, this clear and almost transparent area gradually enlarges downward, although the fungus shows little or no corresponding penetration. At the expiration of three weeks or a month, there is a very distinct, clear, and nearly transparent zone in the medium below the region occupied by the fungous mycelium. Since one could see very distinctly how far the fungous filaments had penetrated into the substrate, it was very evident that the cyto-hydrolytic enzyme had diffused beyond the limits of the mycelium.

The method employed in this investigation for the demonstration of cellulase was the same as that used by Kellerman in his recent work (31) and was utilized to demonstrate the fact that the cyto-hydrolytic enzyme secreted by this fungus penetrates the substrate considerably beyond the limits of the filaments themselves. Tubes containing cellulose agar, in which the fungus had been growing for four weeks, were disinfected externally by washing with a bichloride of mercury solution, and cut off at a point about 12 mm. below the clear portion of the medium. The cotton plug was then flamed and pushed into the tube with a glass rod until the agar was partially shoved out of the cut end of the tube. The clear portion of the agar was then cut into disks about 12 mm. in thickness, which were laid on plates poured with nutrient cellulose agar, great care, of course, being exercised throughout the operation to maintain aseptic conditions. The plates so prepared were then placed in an incubator at 25°C. where they remained for two weeks, at the expiration of which time the cellulose was very distinctly hydrolyzed in a ring about the sterile slices of agar. Microscopic examination confirmed the macroscopic observation that these agar disks were free from any infection.

As might be expected, the activity of the secretion of the enzyme cellulase is influenced by temperature, a fact which is well illustrated by the following experiment: Tubes containing cellulose agar inoculated with the brown-rot fungus were kept at temperatures of 10–12, 16–20, and 24–26°C. respectively, and at the end of twelve days the following results were noted: In the cultures maintained at 10–12°C. no apparent growth or

hydrolysis had taken place; those kept at 16–20°C. showed a good growth but no visible cellulose hydrolysis; and in those maintained at 24–26°C. there was about the same extent of growth as in the preceding series but accompanied by a very evident cellulose hydrolysis, a distinctly clear zone of dissolved cellulose surrounding the region occupied by the fungous mycelium. It is therefore evident that even with approximately the same amount of growth cellulose hydrolysis is much more rapid at the higher temperature.

An effort was made to determine whether or not it is possible to "train up" more active cyto-hydrolytic strains of the *Sclerotinia* and *Penicillium* in question. On the one hand, these fungi were grown for several successive generations on peach-juice agar—a medium in which the organisms show no cytolytic activity. On the other hand, these fungi were cultivated for several successive generations on paper-cellulose agar—a medium which is low in soluble carbohydrates, and one in which the fungi exhibit considerable cytolytic activity. Tubes of paper-cellulose agar were then inoculated with the fungi grown in these two ways and careful observations were made to detect any differences in cyto-hydrolytic activity. No differences developed, however, from which it would appear that the source of cultures of *Sclerotinia* or of *Penicillium* does not materially affect the cellulose-dissolving capacity of these organisms, i. e., each fungus shows the same cellulose-hydrolyzing power whether the organism was cytolytically active during the immediately preceding generations or not.

EFFECT OF THE FUNGUS ON PECTIC SUBSTANCES

The power of organisms to change pectic substances has been considered an important factor in the disintegration and softening of host tissue by certain plant parasites. Before entering into a discussion of the experimental phases of this subject, it will perhaps be well to give some idea of the present status of this question, as well as a very brief resumé of the extensive literature which has accumulated about it.

Fremey (23, 24), in 1840, was the first to report an enzyme acting on pectic substances. This enzyme, which he isolated and called pectase, induced the coagulation of pectin, Fremey attrib-

uting this action of the enzyme to the presence of calcium salts. It is of interest to note that pectase was one of the first plant enzymes to be described. Bertrand and Mallèvre (7, 8) concluded that pectose and pectase are almost universally present in green plants, being especially abundant in the leaves. These authors showed that acidity is an important factor in the inhibition of coagulation of pectic bodies by pectase, and also that either barium, calcium, or strontium is necessary for the action of pectase.

Mangin (35, 36), by microscopic tests, has thrown much light on the nature of the middle lamella and holds that pectose is very pronounced in the cell walls of young tissue. In the older cell walls, on the other hand, this author believes that calcium pectate predominates in the middle lamella, considering that the latter is largely if not entirely composed of this substance and that it frequently collects on the surface of the cell walls adjoining intercellular spaces. Bourquelot (11), and Bourquelot and Hérissey (12) secured a thermo-labile enzyme from barley malt extract which acted upon a solution of pectin (taken from the gentian root), changing the latter in such a way that it was no longer coagulated by pectase. The action of this enzyme, which they called pectinase, was thought by them to be that of converting the pectin into reducing sugar. They also designated as pectinase an enzyme which dissolves the pectic coagulum (the latter has been supposed to be calcium pectate). A good resumé of the status of the chemistry of pectic substances is given by Bigelow and others (10).

A number of investigators have reported upon the action of bacteria on plant cells, including the effects of the organisms on the middle lamella. Winogradsky (55), Behrens (5), and others attributed the changes taking place in the flax plant during retting to the dissolving action which the bacteria exert on the middle lamella. It will be unnecessary to review here any more of the earlier work which has been done along this line, since it has been so thoroughly discussed in the comprehensive publications by Jones (29), and Jones, Harding, and Morse (30) on the soft rot of vegetables. These authors studied the effect of the soft-rot bacillus (*Bacillus carotovorus*) on the host and find that the organism is identical with what has been

designated as *B. oleraceæ* Harrison, and *B. omnivorous* Van Hall, and that it may possibly be identical also with Potter's *B. destructans*. By many tests Jones has shown that this organism secretes an enzyme which causes the disintegration of the host cells by dissolving the middle lamella, which, according to the majority of investigators, is composed of salts of pectic acid. This author has further isolated from pure cultures of the organism an extra-cellular enzyme, which he designated pectinase, that destroys the middle lamella of the cells just as does the growing organism. Jones, therefore, considers this enzyme responsible for the disintegrating action of the bacillus.

In my own work I shall adopt the nomenclature used by Jones (29, 30) and Euler (21), namely, employing pectinase as the term to designate the enzyme inducing coagulation of a pectin solution and also the hydrolysis of calcium pectate, or pectinate.

Methods.—In order to determine the effect of the fungus on the middle lamella I have used two methods, (1) a microscopic study of the effect of the fungus on the host cells, and (2) a study of the effect of the organism on the substances (isolated from the host) which are commonly reported to be constituents of the middle lamella. The first method has been discussed above and may be dismissed here by stating that it yielded no positive evidence that the fungus dissolves the middle lamella. By the second method the problem was studied by isolating pectin from the host and studying the effect of the fungus on it and also on its salts, as, for instance, calcium pectinate.

Pectin was isolated from plums by the following method: Thoroughly ripe fruits were steamed—no water being added, the juice filtered off and treated with Almen's reagent[1] (to precipitate the protein) and with a very dilute solution of oxalic acid (to precipitate the calcium). It was found that under these conditions neither a calcium nor a protein precipitate was thrown down either by Almen's reagent or the oxalic acid, and this procedure, therefore, was deemed unnecessary and was abandoned. The plum juice was carefully filtered through a Buchner filter

[1]Abderhalden, E. Handbuch d. biochem. Arbeitsmethoden **2**: 391–92. 1910. Almen's tannic acid solution is made by treating 4 grams of tannic acid with 8 cc. of a 25 per cent solution of acetic acid, and making up to 190 cc. with 40 or 50 per cent alcohol.

and the filtrate treated with 95 per cent alcohol until a flocculent coagulum of pectin was produced. This pectin was separated by means of a Buchner filter, redissolved in water, reprecipitated with alcohol, again separated by means of a Buchner funnel, and finally dried at a temperature slightly higher than room temperature,—the reprecipitation being for the purpose of purification. It should be noted here that the plums were sufficiently acid to make the addition of hydrochloric acid to the alcohol unnecessary.

Experiments with pectin and pectinase.—From the pectin isolated by the above method a saturated aqueous solution was prepared—some of the mineral nutrient solution[1] minus calcium being added, and the resulting solution rendered sterile by fractional sterilization. Test-tubes of this pectin solution were inoculated with *Sclerotinia cinerea* and *Penicillium expansum* with the result that both organisms produced a rather vigorous growth of mycelium and a few spores. At the expiration of one week the inoculated tubes showed a slight clear area just below the fungous felt due to the coagulation and settling out of the pectin in that part of the solution. The coagulation was at this time somewhat more pronounced in the *Penicillium* cultures than in those of *Sclerotinia*, yet very noticeable in both cases, beginning directly below the fungous felt and progressing toward the bottom of the tube. After two weeks the greater part of the pectin solution was coagulated, the flocculent coagulum, or precipitate, being very different from the precipitate produced in a pectin solution by a calcium salt. It should be emphasized here that every precaution was taken to maintain a calcium-free solution, and when it is considered that the addition of calcium develops a reaction very different from that produced by the enzyme, and, furthermore, that the check gave no coagulation whatever, not even when allowed to stand a month or more, the conclusion would seem to be warranted that calcium is not necessary for the production of a gel by pectinase. Both *Sclerotinia* and *Penicillium*, therefore, produced a coagulum in an aqueous solution of pectin, while no such results were obtained in the controls, thus justifying the conclusion

[1]Nutrient solution employed was the same as mineral nutrient solution A used in preparing cellulose agar, but without the calcium.

that these two fungi are capable of producing pectinase. The cultures were kept at a temperature of 18–20°C.

Experiments with calcium pectinate.—Calcium pectinate was prepared by treating a water solution of pectin with freshly-made limewater (care being exercised to avoid an excess of lime), the product thus obtained being filtered off and thoroughly washed until it was no longer alkaline. The calcium pectinate thus prepared was used in making a pectinate agar in a manner similar to that employed in the preparation of cellulose agar, the same mineral nutrient solution (nutrient A) being used and the whole rendered sterile by fractional sterilization. After the last heating, care was taken to distribute the pectinate, which quickly settles to the bottom of the tubes, uniformly throughout the agar by stirring the medium with a sterile glass rod. These tubes were then inoculated with *Sclerotinia* and with *Penicillium*, the object being to compare the action toward pectic substances of two fungi that have entirely different effects on the host cells, the former producing no softening effects, while the latter causes a very rapid softening and disorganization of the host tissue.

The inoculated tubes of pectinate agar prepared by the above method were kept at a temperature of 22–24°C. Contrary to expectations, there was very little growth when no soluble carbohydrate was supplied, and, furthermore, no dissolving action on the calcium pectinate. On the other hand, when 0.5 per cent glucose was added, both fungi produced a vigorous growth, but neither one gave any indication of pectinate hydrolysis, or dissolution. Here again, as in the cellulose hydrolysis, the two fungi, *Sclerotinia* and *Penicillium*, behave alike. This is not in accordance with the observed behavior of these two organisms toward the host tissue.

ACID RELATIONS OF THE FUNGUS

Some investigators have held that the content of tannin (47) and of malic and other acids of the host determines whether or not the fungus can grow in the tissues and rot the fruit. In accordance with this view a fungus may not so readily attack green as ripe fruit, the former being supposed to exhibit a higher

content of these restraining agents. The question of the acid relation of the host tissue is one of fundamental significance and one that is worthy of considerable investigation; it is important to know to what extent acidity may be a limiting factor in parasitism.

A case in which a certain acid content is favorable for the fungus is developed by Falck (22). He finds the acidity of the substrate to be a conditioning factor for the growth of several species of *Merulius*. In this connection the author observes that *Coniophora*, in particular, acts to pave the way for *Merulius* in that the former organism renders the nutrient substrate decidedly acid, and thereby provides favorable conditions for the germination of the spores and the subsequent growth of mycelium and fruit bodies of *Merulius*. In connection with the investigation of the plum disease here discussed it would be well to know if the acidity of the fruit changes during the progress of its growth, and if so in what direction. It is also essential to know whether or not a change in the acidity of the host can account for the fact that ripe fruit is more susceptible to the disease than green fruit. Some experiments were planned, therefore, to determine to what extent the acidity of the host influences the attack of the parasite, and also to investigate what effects, if any, the fungus has with respect to the acid content of the host.

In order to determine the changes in acidity which take place during the growth of the fruit (plums), several analyses for acidity were made at intervals during the summer. The plums for all of the analyses were taken from the same tree, a known weight of pulp being ground up in a mortar and squeezed through muslin. The acidity was reckoned in the number of cc. of N/10 NaOH required to neutralize one gram of plum pulp. The results were as follows:

June 28, 1 gram plum pulp required 0.66 cc. N/10 NaOH for neutralization,

Aug. 2, 1 gram plum pulp required 2.12 cc. N/10 NaOH for neutralization,

Aug. 19, 1 gram plum pulp required 2.46 cc. N/10 NaOH for neutralization,

the fruit being market ripe on August 19. In these tests my results agree with those obtained by Bigelow and Gore (10) for peaches, and with those of Thompson and Whittier (49) for some other fruits. The last mentioned investigators, however, found that the acidity of peaches decreases toward maturity. I have been unable to secure data covering the acidity of plums throughout the season.

The above results show that the acid content of plums increases rather than diminishes toward the maturity of the fruit. The results of the experiments and field observations show that mature and ripe fruit is much more susceptible than the green and immature fruit. The above facts, showing that as the fruit approaches maturity the acidity increases while the susceptibility to the disease also increases, indicate that there is no close relationship between the low acid content of the host and susceptibility to the brown-rot fungus, and that we must look to other factors to explain infection as observed in the field. As pointed out, my experiments indicate that penetration is a

TABLE III

RELATION OF THE GROWTH OF SCLEROTINIA CINEREA TO THE REACTION OF THE MEDIUM

Medium	Acidity	Growth after 8 days	Growth after 16 days	Spore production
Cherry juice	+2.3*	−†	+†	+
Cherry juice	+1.5	++	++	++
Cherry juice	+1.0	+++	++	++
Cherry juice	+0.15	−	+	+
Cherry juice	−0.15	0	++	0
Cherry juice	−0.30	0	++	0

*Acidity is given in cc. of N/10 NaOH necessary to neutralize 1 cc. of the juice.

†The + sign indicates a fairly good mycelial growth, or spore formation, and the − sign indicates that the growth was just perceptible; 0 indicates no growth, or no spore formation.

very important factor. It is possible that a study of the tannin content[1] might yield some relation of interest.

A preliminary experiment was planned to determine the acidity at which the optimum growth and spore production of the fungus occurs. For this purpose the juice from ripe sour cherries was used. The juice was squeezed out of the cherries (no water being added) and a portion titrated to determine the acidity. Then 50 cc. of this liquid were put into each of a number of Erlenmeyer flasks of 125 cc. capacity; some of the flasks were left untreated, while others received various quantities of N/10 NaOH to bring each to the desired acidity or alkalinity. The flasks were then sterilized and inoculated. The results are given in table III.

It is clear, therefore, that although the fungus eventually grows on a medium as acid as the natural juice of sour cherries, it grows more luxuriantly on a somewhat less acid medium. It is a rather significant fact that on the media near the neutral line the fungus at first shows no perceptible growth, but at the expiration of two weeks has produced nearly as much mycelial growth as on the acid medium. It is also of interest to note that we find spore formation abundant on the very acid media but entirely lacking on the alkaline media. This experiment indicates that the fungus can adjust itself to a slight degree of alkalinity.

OXALIC ACID PRODUCTION BY THE FUNGUS

The first important reference to oxalic acid production by fungi is in the publication by de Bary reviewed in a preceding section. He reports that the older hyphæ of the fungus were encrusted with crystals of oxalic acid, and he attributed some of the poisonous action of the parasite to the production of this substance; in fact, he mentions oxalic acid fermentation. Since the appearance of de Bary's paper a limited number of investi-

[1] Cook and Bassett and their associates (17) believe that there are enzymes in the host plant which may act upon cell constituents and play the rôle of alexins. They are of the opinion that tannin, as such, is not abundant in fruits, but that it may be formed by the action of oxidizing enzymes upon certain phenols. Injuries produced by parasitic fungi may accelerate the activity of the host in the production of tannin, the latter perhaps being toxic to the growth of parasitic fungi.

gators have reported the presence of oxalic acid resulting from the growth of both fungi and bacteria, but unfortunately much of this work is of little value, because methods of analysis are not given. The detection of this acid by some methods is very unsatisfactory.

A few years after de Bary's work, Wehmer (54) published an extensive series of articles on this subject. He studied a number of fungi (mostly saprophytic) with reference to oxalic acid excretion, and of these he found *Aspergillus* to be the most active and *Penicillium* next, and, therefore, he confined his studies to these two fungi. Some of the factors concerned in the production of oxalic acid or its salts, according to Wehmer, may be summed up here: (1) A large yield of oxalic acid is not produced in the presence of free organic or inorganic acids, not being found in the medium when free acids exceeded 0.2–0.3 per cent, while, on the other hand, it can be formed in the presence of as much as 2–3 per cent of the salts of these acids. (2) The sources of nitrogen are very important, for the amount of the oxalic acid produced varies according to the kind and quantity of nitrogenous compounds supplied. (3) Abundant oxalic acid formation is favored by the addition of some basic phosphate, or at least some compound with which the acid can combine to form a soluble salt. (4) The effect of light or darkness on oxalic acid formation is inappreciable. (5) Temperature is an influencing factor in oxalate production, for the latter is inhibited by a high temperature, the temperature for a maximum oxalate production being, in fact, very near the minimum for the growth of the organism.

Wehmer's analytical method consisted in precipitating out the oxalic acid, or its soluble oxalate, as the calcium salt, which was filtered off, dried to a constant weight, and weighed. Although this method is perhaps as well suited for this purpose as any other reported, it is open to criticism. A detailed discussion, however, will not be given here.

Wehmer holds that oxalic acid is a type of excretion, and that it is in some way connected with respiration, that is, with CO_2 elimination. He considers that the variability in the amount of oxalic acid produced is due to its use in the metabolism of the fungus. Emmerling (20), in his contribution to this subject,

emphasizes the influence of such nitrogenous substances as proteins, amino acids, and amides in the nutrient. He finds that *Aspergillus niger* when grown in non-amino acids, for example, tartaric, lactic, etc., produces no oxalic acid, whereas an abundant oxalic acid production results on such substances as peptone or aspartic acid.

Smith (46) and Peltier (41) both conducted experiments to determine whether or not oxalic acid is present in media in which *Botrytis* has been growing. Peltier reported negative results, but Smith found oxalic acid and thinks that the poisoning effect of the fungus is perhaps due to the presence of this acid. Unfortunately, neither of these authors gives his methods of analysis, and, with the exception of one incident in Smith's publication, the quantity of oxalic acid found is not reported. Peltier and others have been able to produce an injury with oxalic acid similar to that produced by certain parasitic fungi, such as *Botrytis*, yet this is not conclusive evidence that oxalic acid is the toxic substance secreted by the organism.

The articles mentioned above constitute the chief publications that have to deal with the production of oxalic acid by fungi. The publications on the production of oxalic acid by bacteria and other plants will not be reviewed here. Whether oxalic acid production is a phenomenon peculiar to certain genera or to certain species of the fungi, whether it is purely the result of external conditions, or whether it results primarily from certain constituents of the medium, has not been clearly demonstrated. A series of experiments was planned in the hope of throwing some light on its production in the fungus here studied.

The method of analysis employed was a modification of Wehmer's method of precipitating the oxalate with calcium chloride and determining the amount of oxalate thus precipitated. This method, however, is not well adapted to the purpose at hand, especially when quantitative methods are used, and fruit juice is employed for the medium on which to grow the fungus. An attempt is being made to develop a method that will be better suited to our purpose.

Culture media were prepared from peaches and plums by filtering the juices of these fruits through a Hill pressure filter under sterile conditions. The product thus obtained was

placed in flasks and incubated for a week and found to be sterile, after which the flasks were inoculated with *Sclerotinia cinerea*. At the expiration of thirty-seven days these cultures were analyzed and were found to contain the following amounts of oxalic acid per 50 cc. of the respective juices:

Plum juice..... 0.0019 grams of oxalic acid,
Peach juice..... 0.0077 grams of oxalic acid,
Peach juice..... 0.0094 grams of oxalic acid,
Control..... No trace of oxalic acid.

Plum and peach juices that had been sterilized by heat, thereby precipitating some of the contained proteinaceous material, were also used as culture media, and here, too, every culture containing the fungus gave a positive test for oxalic acid.

For investigating the production of oxalic acid by the fungus in the unaltered fruit, lots of 500 grams each of peaches were disinfected with bichloride of mercury solution, inoculated respectively with *Sclerotinia*, *Penicillium*, and *Aspergillus niger*, and kept under sterile conditions until the fruits were decayed, or, in the case of the *Penicillium* and *Aspergillus*, until partially decayed. The decayed fruits were then digested with hydrochloric acid and analyzed for their oxalic acid content with the following results:

Peach inoculated with *Penicillium* . . No trace of oxalic acid,
Peach inoculated with *Aspergillus*. . . No trace of oxalic acid,
Peach inoculated with *Sclerotinia cinerea*.....
0.0087 grams of oxalic acid,
Peach control..... No trace of oxalic acid.

The results of these experiments with oxalic acid show that *Sclerotinia cinerea* when grown either on fruit juices or on peaches produces more or less oxalic acid as a result of its metabolism. It is also significant that the other two fungi employed, namely, *Aspergillus* and *Penicillium*, which are not natural parasites on the plum or the peach, produced no oxalic acid under the conditions in which the experiments were carried out.

SUMMARY

1. The brown-rot organism will infect fruits which are immature, even penetrating those which are not more than half-grown or those in which the pits are still soft, provided the

skin is punctured. Infection of green fruits is also effected when a portion of the mycelial felt of the fungus is laid on the surface of the plum. On the other hand, ripe or nearly mature fruits may be readily inoculated by sowing a spore suspension on the unpunctured surface.

2. The fungus does not show any particular affinity for the middle lamella, but penetrates and permeates with equal avidity any part of the host tissue.

3. A study of the effect of the organism on the host gives no positive evidence that a toxic substance is abundantly secreted in advance of penetration.

4. The fungus shows very slight cytolytic action with respect to cellulose isolated from the plum, while, on the other hand, the organism readily hydrolyzes cellulose from filter paper when this is the only carbohydrate supplied. No general cytolytic action of the organism on the cell wall of the host is perceptible.

5. An aqueous solution of pectin isolated from plums was coagulated by *Sclerotinia*, thus indicating the secretion of the enzyme pectinase. In respect to its action on pectic substances, *Sclerotinia cinerea* behaves in a manner similar to that of *Penicillium expansum*, yet these two organisms produce very different effects on the host, the former producing a firm rot and the latter a soft one. Neither organism will dissolve calcium pectinate.

6. The experiments on the acid relations of the fungus indicate that the changing acidity of the host as the fruit reaches maturity does not explain the fact that ripe fruit is more susceptible to the disease than green fruit.

7. The brown-rot fungus produces oxalic acid when grown either on a fruit juice medium or on peaches.

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BIBLIOGRAPHY

1. de Bary, A. Recherches sur le développement de quelques champignons parasites. Ann. d. Sci. Nat. Bot. IV. **20**: 5–148. 1863.
2. ———, Neue Untersuchung ueber Uredineen II. Monatsber. d. Akad. d. Wiss. z. Berlin. 1886.
3. ———, Morphologie und Physiologie der Pilze, Flechten, und Myxomyceten. Handbuch der physiologischen Botanik **2**: 1–316. 1866. [cf. pp. 212–19.]
4. ———, Ueber einige Sclerotinien und Sclerotienkrankheiten. Bot. Zeit. **44**: 377–87, 393–404, 409–26, 433–41, 449–61, 465–74. 1886.
5. Behrens, J. Untersuchungen über die Gewinnung der Hanffaser durch natürliche Röstmethoden. Centralbl. f. Bakt. II. **8**: 114–20, 131–37, 161–66, 202–10, 231–36, 264–68, 295–99. 1902.
6. ———, Beiträge zur Kenntnis der Obstfäulnis. Centralbl. f. Bakt. II. **4**: 514–22, 547–53, 577–85, 635–44, 700–706, 739–46, 770–77. 1898.
7. Bertrand, G., et Mallèvre, A. Recherches sur la pectase et sur la fermentation pectique. I. Jour. de Bot. **8**: 390–96. 1894.
8. ———, ———, Sur la diffusion de la pectase dans le règne végétal et sur la préparation de cette diastase. Jour. de Bot. **10**: 37–41. 1896.
9. Biffen, R. H. On the biology of Bulgaria polymorpha, Wett. Ann. Bot. **15**: 119–34. *pl. 7*. 1901.
10. Bigelow, W. D., Gore, H. C., and Howard, B. J. Studies on apples. U. S. Dept. Agr., Bur. Chem. Bul. 94: 1–99. *pl. 1–5*. 1905.
11. Bourquelot, Em. Sur la physiologie du gentianose; son dédoublement par les ferments solubles. Compt. rend. acad. Paris **126**: 1045–47. 1898.
12. ———, et Hérissey, H. Sur l'existence dans l'orge germée d'un ferment soluble agissant sur la pectine. Compt. rend. acad. Paris **127**: 191–94. 1898.
13. ———, ———, Les ferments solubles du Polyporus sulfureus (Bull.). Bull. Soc. Myc. Fr. **11**: 235–39. 1895.
14. Buller, A. H. R. The enzymes of Polyporus squamosus, Huds. Ann. Bot. **20**: 49–59. 1906.
15. ———, The destruction of wood paving blocks by Lentinus lepideus. Fr. Jour. Econ. Biol. **1**: 1–10. *pl. 1–11*. 1905.
16. Büsgen, M. Ueber einige Eigenschaften der Keimlinge parasitischer Pilze. Bot. Zeit. **51**[1]: 53–72. *pl. 2–3*. 1893.
17. Cook, M. T., Bassett, H. T., Thompson, F., and Taubenhaus, J. J. Protective enzymes. Science N. S. **33**: 624–29. 1911.
18. Czapek, F. Zur Biologie der holzbewohnenden Pilze. Ber. d. deut. bot. Ges. **17**: 166–70. 1899.
19. Dox, A. W. The intracellular enzyms of Penicillium and Aspergillus. U. S. Dept. Agr., Bur. Pl. Ind. Bul. 120: 1–70. 1910.
20. Emmerling, O. Oxalsäurebildung durch Schimmelpilze. Centralbl. f. Bakt II. **10**: 273–75. 1903.
21. Euler, H. General chemistry of enzymes. 1–319. 1912.
22. Falck, R. Hausschwamforschungen. Zeitschr. f. Forst. u. Jagdwesen. Heft 6. p. 1–405. *pl. 1–17*. 1912. [cf. pp. 273–80.]
23. Fremey, E. Premiers essais sur la maturation des fruits. Recherches sur la pectine et l'acide pectique. Jour. de Pharmacie **26**: 368–93. 1840. [Original not consulted.]

24. Fremey, E. Memoire sur la maturation des fruits. Ann. Chim. et Phys. III. **24**: 1–58. 1848. [Original not consulted.]
25. Fulton, H. R. Chemotropism of fungi. Bot. Gaz. **41**: 81–108. 1906.
26. Hartig, R. Die Zersetzungserscheinungen des Holzes der Nadelholzbäume und der Eiche. Berlin. 1–151. *pl. 1–21*. 1878.
27. Humphrey, J. E. On Monilia fructigena. Bot. Gaz. **18**: 85–93. *pl. 7*. 1893.
28. Van Iterson, C., Jr. Die Zersetzung von Cellulose durch aërobe Mikroorganismen. Centralbl. f. Bakt. II. **11**: 689–98. *pl. 1*. 1904.
29. Jones, L. R. The cytolytic enzyme produced by Bacillus carotovorus and certain other soft rot bacteria. Centralbl. f. Bakt. II. **14**: 257–72. 1905.
30. ———, Harding, H. A., and Morse, W. J. The bacterial soft rots of certain vegetables. I. N. Y. (Geneva) Agr. Exp. Sta. Tech. Bul. 11: 251–368. 1909. [cf. pp. 291–368.] *Ibid*, Vermont Agr. Exp. Sta. Bul. 147: 243–360. 1910. [cf. pp. 283–360.]
31. Kellerman, K. F. The excretion of cytase by Penicillium Pinophilum. U. S. Dept. Agr., Bur. Pl. Ind. Circ. 118: 29–31. 1913.
32. ———, and McBeth, I. G. The fermentation of cellulose. Centralbl. f. Bakt. II. **34**: 485–94. *pl. 1–2*. 1912.
33. Kohnstamm, P. Amylolytische, glycosidspaltende, proteolytische und cellulose lösende Fermente in holzbewohnenden Pilzen. Beih. z. bot. Centralbl. **10**: 90–121. 1901.
34. Kühn, J. Die Krankheiten der Kulturgewächse, ihre Ursachen und ihre Verhütung. Berlin. 1–312. *pl. 1–7*. 1858.
35. Mangin, L. Propriétés et réactions des composés pectiques. Jour. de Bot. **6**: 206–12, 235–44, 363–68. 1892.
36. ———, Recherches sur les composés pectiques. Jour. de Bot. **7**: 37–47. *pl. 1.*, 121–31. *pl. 2.*, 325–43. 1893.
37. Matheny, W. A. A comparison of the American brown-rot fungus with Sclerotinia fructigena and S. cinerea of Europe. Bot. Gaz. **56**: 418–32. *f. 1–6*. 1913.
38. McBeth, I. G., and Scales, F. M. The destruction of cellulose by bacteria and filamentous fungi. U. S. Dept. Agr., Bur. Pl. Ind. Bul. 266: 1–52. *pl. 1–4*. 1913.
39. Miyoshi, M. Die Durchbohrung von Membranen durch Pilzfäden. Jahrb. f. wiss. Bot. **28**: 269–89. 1895.
40. Nordhausen, M. Beiträge zur Biologie parasitärer Pilze. Jahrb. f. wiss. Bot. **33**: 1–46. 1899.
41. Peltier, G. L. A consideration of the physiology and life history of a parasitic Botrytis on pepper and lettuce. Rept. Mo. Bot. Gard. **23**: 41–74. *pl. 1–5*. 1912.
42. Reed, H. S. The enzyme activities involved in certain fruit diseases. Ann. Rept. Va. Agr. Exp. Sta. **1911–1912**: 51–77. 1912.
43. Schellenberg, H. C. Untersuchungen über das Verhalten einiger Pilze gegen Hemizellulosen. Flora **98**: 257–308. 1908.
44. Schmidt, E. W. Über den Parasitismus der Pilze. Zeitschr. f. Pflanzenkrankh. **19**: 129–43. 1909.
45. Smith, E. F. Peach rot and peach blight. (Monilia fructigena Pers.) Jour. Myc. **5**: 123–34. 1899.
46. Smith, R. E. The parasitism of Botrytis cinerea. Bot. Gaz. **33**: 421–36. 1902.
47. von Schrenk, H. A disease of the black locust (Robinia pseudacacia L.). Rept. Mo. Bot. Gard. **12**: 21–31. *pl. 1–3*. 1901.

48. Thom, C. Cultural studies of species of Penicillium. U. S. Dept. Agr., Bur. Animal Ind. Bul. 118: 1–109. *f. 1–36*. 1910.
49. Thompson, F., and Whittier, A. C. Fruit juices. Del. Agr. Exp. Sta. Bul. 102: 1–28. 1913.
50. Ward, H. M. On the biology of Stereum hirsutum. Phil. Trans. Roy. Soc. Lond. 189: 123–34. 1897.
51. ————, A lily-disease. Ann. Bot. 2: 319–82. *pl. 20–24*. 1888.
52. ————, Penicillium as a wood-destroying fungus. Ann. Bot. 12: 565–66. 1898.
53. Wehmer, C. Monilia fructigena Pers. (=Sclerotinia fructigena m) und die Monilia-Krankheit der Obstbäume. Ber. d. deut. bot. Ges. 16: 298–307. *pl. 18*. 1898.
54. ————, Entstehung und physiologische Bedeutung der Oxalsäure im Stoffwechsel einiger Pilze. Bot. Zeit. 49: 233–46, 249–57, 271–80, 289–98, 305–13, 321–32, 337–46, 353–63, 369–74, 385–96, 401–7, 417–28, 433–39, 449–56, 465–78, 511–18, 531–39, 547–54, 563–69, 579–84, 596–602, 611–20, 630–38. 1891.
55. Winogradsky, S. Sur le rouissage du lin et son agent microbien. Comt. rend. acad. Paris 121: 742–45. 1895.
56. Woronin, M. Über Sclerotinia cinerea und Sclerotinia fructigena. Mem. de l'Acad. Imp. d. Sci. de St. Petersbourg, Classe Phys. Math. VIII. 10: 1–38. *pl. 1–6*. 1899.
57. Zschokke, A. Ueber den Bau der Haut und die Ursachen der verschwindenen Haltbarkeit unserer Kernobstfrüchte. Landw. Jahrb. d. Schweiz 11: 153–97. *pl. 1–2*. 1897.